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Variable response from abalone larvae (*Haliotis iris, H. virginea*) to a range of settlement cues

Rodney D. Roberts[1,2]
and
Christine M. Nicholson[1]
[1]Cawthron Institute, Private Bag 2, Nelson, New Zealand
[2]University of Otago, P.O.Box 56, Dunedin, New Zealand

Abstract

We exposed competent larvae of two abalone species (*Haliotis iris* and *H. virginea*) to a range of potential settlement cues, and observed a hierarchy of responses. The end point and the timing of the settlement process varied, depending on the cue provided. Response was qualitatively consistent between experiments, but showed some quantitative variation. Rocks coated with crustose coralline algae (CCA) induced 80–100% of larvae to attach and metamorphose, and 50–100% to grow peristomal shell, within 2 days. A distilled water extract of CCA induced almost 100% attachment within 2 days, but metamorphosis occurred gradually between 2 and 5 days. Shell growth was minimal, apparently inhibited by chemical interference from the extract. γ-Aminobutyric acid (GABA, 1 μM) induced 90–100% attachment and 20–60% metamorphosis in *H. iris* within 2 days. For *H. virginea,* 1 μM GABA induced attachment (65–100%) but typically only 0–5% metamorphosis within 2 days. KCl (10 mM) added to seawater induced attachment (50–70%) but less than 10% metamorphosis. Different diatoms induced responses ranging from rapid or gradual induction of metamorphosis, to minimal response. Some combinations of cues produced synergistic effects. *E.g.*, GABA (1 μM) plus diatom biofilm induced more metamorphosis (49%) than biofilm (28%) or GABA (1%) alone. Addition of CCA crude extract to the same biofilm hastened metamorphosis. Observations of attachment without subsequent metamorphosis suggest that there may be separate cues for attachment and metamorphosis. This study highlights the complexity of the settlement response in abalone.

Introduction

Most marine invertebrates have a free swimming larval stage prior to a benthic adult phase. For many species, including abalone, the transition from swimming larva to crawling post-larva (herein referred to as "settlement") requires a chemical cue (Crisp 1974; Pawlik 1992). Larval abalone settle and metamorphose in response to various substances including intact crustose coralline algae (CCA), extracts from CCA (Morse *et al.* 1980), γ-aminobutyric acid (GABA – Morse 1984; Yang and Wu 1994), excess potassium ion (Yool *et al.* 1986; Yang and Wu 1995) and cultures of benthic diatoms (Kawamura and Kikuchi 1992).

Some variations in the settlement response of abalone have been documented, *e.g.* Searcy-Bernal *et al.* (1992) found that a diatom-based biofilm induced metamorphosis more slowly than 1 μM GABA or mucus treatments; and Morse (1984) reported that concentrations of GABA exceeding 1 μM induced attachment, but inhibited metamorphosis. However, there has been little detailed comparison of settlement responses across a range of different cues. Such a comparison needs to account for variation between batches of larvae (Dineen and Hines 1994) and differences in methods between

experiments (Morse 1992). One aim of this study was to determine the response of a single batch of abalone larvae to several cues which have previously been shown to induce settlement in other abalone (see above). Another was to quantify the extent of inter-experiment variability (using standardised methods) for a subset of the cues.

Variation in the timing or completeness of the settlement response is important in abalone culture. Mortality during the first two months is typically 85–100% (Searcy-Bernal *et al.* 1992), and failure to metamorphose can be an important factor (Ebert and Houk 1989). The speed of the settlement response, the proportion of larvae responding, and the distinction between attachment and metamorphosis are all important in managing the settlement process in commercial hatcheries.

Anecdotal reports of larvae of *H. australis* Gmelin 1791 attaching, but later resuming swimming (G. Moss, pers. comm.), suggest that attachment and metamorphosis may be uncoupled, and triggered by different cues. By quantifying the components of the settlement response separately over time, we hoped to gain insights into the cue(s) for attachment, metamorphosis and post-larval development.

This paper describes the timing and end point of the settlement response to a range of cues, using two New Zealand abalone species – *Haliotis virginea virginea* Gmelin 1791 and *H. iris* Gmelin 1791.

Materials and methods

Larval rearing

Spawning was induced by adding hydrogen peroxide and sodium hydroxide (final concentrations of 6 mM and 1 mM respectively) to the seawater bathing ripe adults (Morse *et al.* 1977). Larvae were hatched and reared in 1 μm filtered, flowing, natural seawater. The rearing system was cleaned, and the filters were changed every 2 days. *H. virginea* were reared at the Glenhaven Aquaculture Centre Ltd in Nelson. *H. iris* larvae were initially reared at Rainbow Abalone Ltd in Taranaki, or the National Institute of Water and Atmospheric Research in Wellington. Operculate larvae were transported to Nelson using established techniques (Tong and Moss, 1992) then reared at the Glenhaven Aquaculture Centre until used in settlement bioassays. Larval stage was determined by examining radula development (Tong and Moss 1992). Larvae gained competence to settle at ca. ~100°C.days of age, which corresponded to five rows of fully chitinized radula teeth (Roberts, unpubl. for *H. virginea*; Moss and Tong (1992) for *H. iris*).

Settlement cues

Pebbles coated with CCA (>90% cover of "encrusting" to "warty" growth form (Shepherd and Daume 1996) species unknown) were collected from Cable Bay, Nelson (173°24'E, 41°11'S) and held in flowing raw sea water until assayed. Crude extract of CCA was prepared by soaking CCA-coated rocks in distilled water for 36 hours. Extract (unfiltered) was evaporated to dryness at 35°C, resuspended in distilled water and stored at –20°C. Extract was assayed at 0.44 mg dry extract per ml of assay medium for *H. virginea*, but at 0.22 mg/ml for *H. iris* because the higher dose was mildly toxic. A partially purified extract was prepared by anion exchange chromatography. Crude extract was buffered at pH 6.0 with 20 mM Bis-Tris (Sigma), filtered (0.45μm, Millipore HAWP) then pumped through a column of Q-Sepharose Fast Flow (Pharmacia, 12 cm x 2.5 cm diam.) previously equilibrated with 20 mM Bis-Tris, pH 6.0. Bound material was eluted with 2.0 M NaCl, then bioassayed as described below. Active fractions were pooled, and stored at –20°C. This pooled sample is referred to as "partially purified extract" below.

Potassium chloride (KCl, 10 mM, Baker) was made up in 1 μm filtered, UV-treated natural seawater ("FSW"). A frozen stock solution of 10 μM GABA (Sigma) in distilled water was diluted with FSW to a final concentration of 1 μM. The salinity change caused by the distilled water did not affect the larvae. FSW was run as a negative control.

Benthic diatoms were isolated from Nelson coastal waters (*Nitzschia* sp. 1, *Nitzschia* sp. 2, *Navicula* sp. 1) or obtained from the Tohoku National Fisheries Research Institute in Japan (*Cocconeis scutellum* var. *parva* Cleve 1895). Diatoms were cultured in F/2 medium (Guillard 1975) in tissue culture dishes (Falcon 3043) on a 12:12 LD cycle at 30–70 $\mu E.m^{-2}.sec^{-1}$. The diatoms and associated microbes formed a film on the floor and walls of the chambers. Diatom cell density was determined by replicate counts of the chamber floor immediately prior to assays. Cultures were not axenic. For settlement bioassays, F/2 growth medium was replaced with FSW, and larvae were added to the culture chamber.

Larval settlement bioassays

For settlement bioassays, competent larvae were transferred to 12 well tissue culture dishes (Falcon 3043) at densities of ca. 50–100 per well. Assays were run in FSW containing 150 µg/ml each of Penicillin G sodium (Biochemie) and Streptomycin sulphate BP (Sigma), total assay volume 3.37 ml. These antibiotics ensured virtually nil mortality of larvae/post-larvae. Assay dishes were wrapped in foil and incubated at 17 ± 1°C for up to 12 days without water change.

Larval settlement was quantified by inspecting the floor and walls of the assay chamber, and the surface film of the assay water, with inverted and dissecting microscopes. Swimming larvae could not be accurately counted due to their mobility. Where necessary, larvae were killed and counted at the end of the experiment to allow calculation of the number of swimming larvae by subtracting the sum of all non-swimming larvae (at day 2, 4 etc) from the total number of larvae. Abalone attached to pebbles coated with CCA were dislodged with a water jet to allow accurate counting. We calculated the percentage of live animals showing: (a) "shell growth" (velum shed, peristomal shell visible); (b) "metamorphosis" (velum shed, with or without peristomal shell growth); (c) "attachment" (metamorphosed or attached by foot).

Table 1 details the aims of this study, and the larvae and treatments used.

Table 1. Study aims, with details of larvae and treatments used. Number of rows of teeth on radula is a measure of larval development at the time the experiment was set up. Larvae with >5 rows of teeth are competent to settle. n = number of replicate treatments (50–100 larvae per replicate). CCA = crustose coralline algae; FSW = 1 µm filtered, UV-treated, natural seawater.

Aim	Abalone species	Spawning date	Rows of teeth on radula	n	Treatments
Determine response of a single larval batch to a range of settlement cues, and examine differences between the two abalone species	*H. iris* *H. virginea*	12/9/96 5/9/96	10 6	6 5	Intact CCA, CCA extract, GABA (1 µM), KCl (10 mM), FSW control
Determine whether chemical interference from CCA extracts inhibits post-larval shell growth	*H. virginea*	15/7/96	10	Various – see Table 2	Partially purified CCA extract
Demonstrate the range of settlement responses elicited by different diatom-based microbial films	*H. iris* *H. virginea*	12/9/96 1/8/96	10 9	6 4	Cultures of four benthic diatom strains (not axenic)
Examine the potential for enhancing settlement by applying combinations of cues	*H. virginea*	1/8/96	9	4	GABA + *Nitzschia* sp. 1, CCA extract + *Nitzschia* sp. 1
Quantify variation in settlement response between experiments for selected cues	*H. iris* *H. virginea*	Various – see Table 3	7–12 – see Table 3	2–5 – see Table 3	Intact CCA, GABA (1 µM), FSW control

Results

Response to different settlement cues

Complete and rapid settlement was induced by CCA-coated pebbles for both *H. iris* and *H. virginea* (Figure 1a–b). Post-larvae on CCA-coated pebbles fed actively and produced faecal material. In contrast, when larvae were incubated without any cue, less than 10% of larvae attached, and virtually none metamorphosed (Figure 1, i–j).

The response to a distilled water extract of CCA differed in two respects from the response to intact CCA. Firstly, while the extract induced attachment and metamorphosis, it did not induce shell growth (Figure 1c–d). Secondly, metamorphosis was delayed relative to CCA, and occurred largely between 2 and 5 days after introduction of the extract (Figure 1c–d). In extract-induced post-larvae, the mantle margin migrated to the edge of the larval shell, and feeding commenced, but was weak and intermittent. The post-larvae produced no faecal material, and no more than a short stub of peristomal shell. Two lines of evidence suggest that chemical interferences were responsible for the lack of shell growth. (1) When larvae were added to *Nitzschia* sp. 1 with and without CCA extract, mean length of new shell was 3–4 times lower in the presence of crude extract (Table 2, t=4.5, p<0.001). (2) When larvae were incubated with partially purified extract at near its lowest effective dose, significantly more post-larval shell was added than at a four-fold higher concentration (Table 2, t=10.1, p<0.001).

Testing of pure compounds revealed further differences in larval responses. *H. iris* exposed to 1 µM GABA showed high initial attachment (which fell within several days) and moderate percentage metamorphosis (Figure 1e). The resulting post-larvae displayed normal feeding movements despite a virtual absence of particulate food, but did not produce faecal material. Yolk was still visible in post-larvae 10 days after introduction of the GABA. Post-larvae grew 30–120 µm (n=46) of peristomal shell length during 10 days incubation, with most of the growth occurring in the first 4 days. For *H. virginea* the response was different – 1 µM GABA induced attachment but no metamorphosis (Figure 1f).

Potassium chloride (10 mM added to seawater) induced moderate attachment, but very little metamorphosis of either abalone species, even after 11 days of exposure (Figure 1g–h). The percent attachment fell after several days of incubation (Figure 1g–h).

With both KCl and GABA we have tried many variations on our methods (assay chambers up to 400 ml, no antibiotics, different antibiotics, range of KCl/GABA concentrations), but have always obtained results similar to those presented.

Table 2. Comparison of mean (± SE) width of post-larval shell produced by *H. virginea*: (a) on *Nitzschia* sp. 1 (1.1 ± 0.1 x 10^5 diatom cells.cm^{-2}, not axenic) in presence/absence of CCA extract, or (b) in high versus low doses of partially purified CCA extract. Post-larval shell growth was measured as the maximum width of the peristomal shell band. n = number of abalone measured.

Treatment	Post larval shell width (µm)	Days of incubation	n
a) Diatom with/without CCA extract			
Nitzschia sp. 1 with CCA extract (0.44 mg/ml)	16 ± 3.9	10	31
Nitzschia sp. 1 without CCA extract	56 ± 7.9	10	39
b) Partially purified CCA extract			
68µL – high dose	6.0 ± 1.6	6	31
17µL – low dose	26.1 ± 1.2	6	31

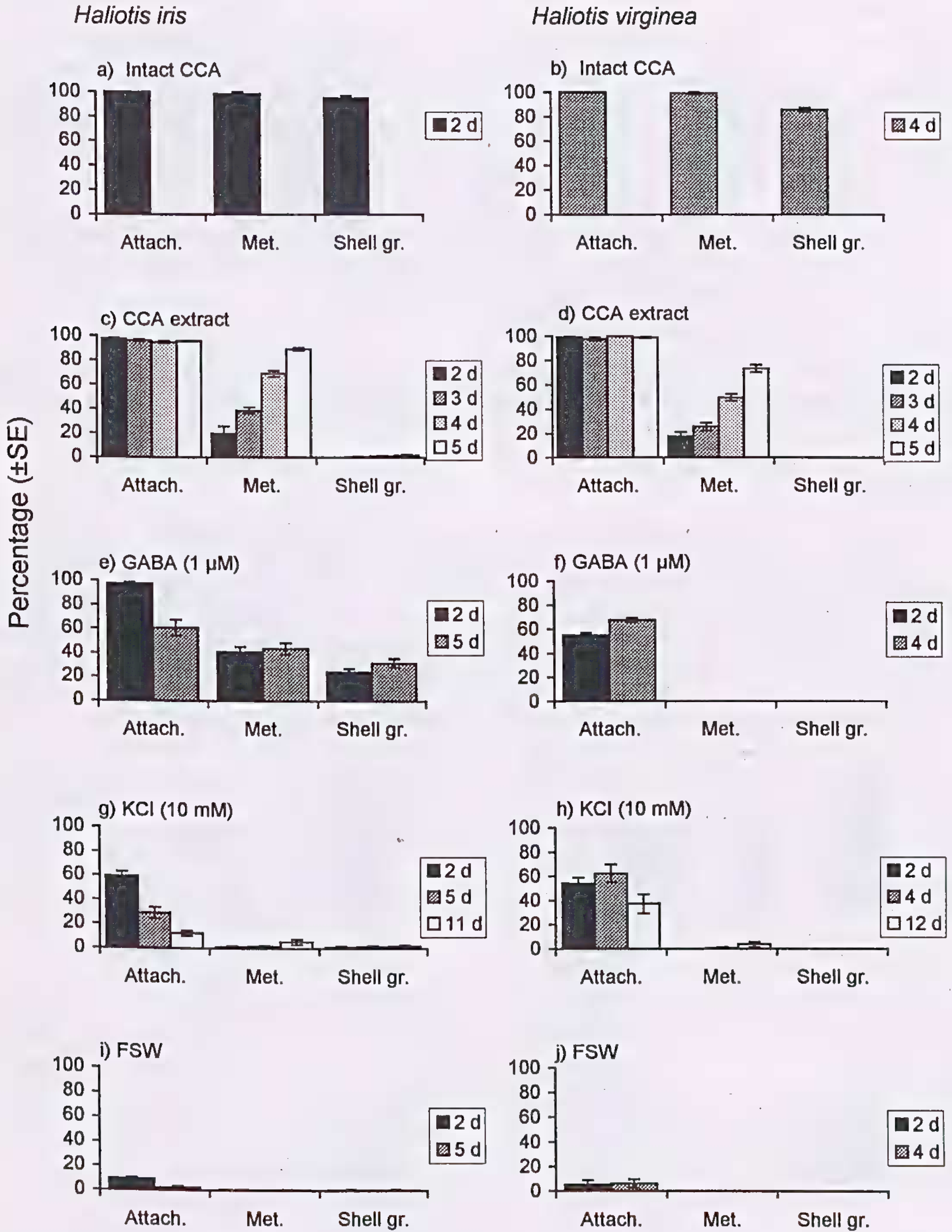

Figure 1. Comparison of the settlement response of abalone larvae (single batch for each species) to four settlement cues and a negative control (FSW). Intact CCA = pebbles covered with crustose coralline algae; CCA extract = distilled water extract of CCA (0.44 mg/ml); FSW = 1 μm filtered, UV-treated, natural seawater. Attach. = attachment, Met. = metamorphosis, Shell gr. = shell growth. Legend gives number of days of incubation at time of counting. *H. iris*: n=6. *H. virginea*: n=5.

Responses to diatom films

Data from four diatom strains (not axenic) are presented to demonstrate that microbial films can elicit the full range of responses described above for other cues. *Nitzschia* sp. 1 induced rapid attachment, metamorphosis and shell growth in *H. iris* (Figure 2a) with effectiveness comparable to intact CCA. *Cocconeis scutellum* var. *parva* also induced all three components of settlement in *H. virginea*, but the onset of metamorphosis and shell growth was gradual, occurring largely after 5–10 days of exposure (Figure 2b). *Navicula* sp. 1 induced moderate percent attachment, but virtually no metamorphosis in *H. virginea* (Figure 2c), echoing the response of this species to 10 mM potassium chloride and 1 μM GABA. *Nitzschia* sp. 2 was a very weak inducer of attachment and metamorphosis in *H. iris* (Figure 2d). Post-larvae which metamorphosed on diatom films showed normal feeding behaviour and produced faecal material.

Synergistic effects between cues

In experiments examining the enhancement of settlement responses by combining stimuli, we have observed some synergistic interactions between pairs of cues, *e.g.* GABA (1 μM) combined with *Nitzschia* sp. 1 induced more metamorphosis in *H. virginea* than the sum of the two cues alone (Figure 3). Addition of CCA extract to a *Nitzschia* sp. 1 culture hastened metamorphosis of *H. virginea* (Figure 4).

Inter-experiment variability in response

In order to examine inter-experiment variability, we compared the settlement response over a series of experiments using standard procedures. Data are presented for three treatments representing nil (FSW), partial (1 μM GABA) and complete (intact CCA) settlement responses. Detailed data (Table 3) are presented for *H. virginea* only, except for GABA, where the response from the two species differed.

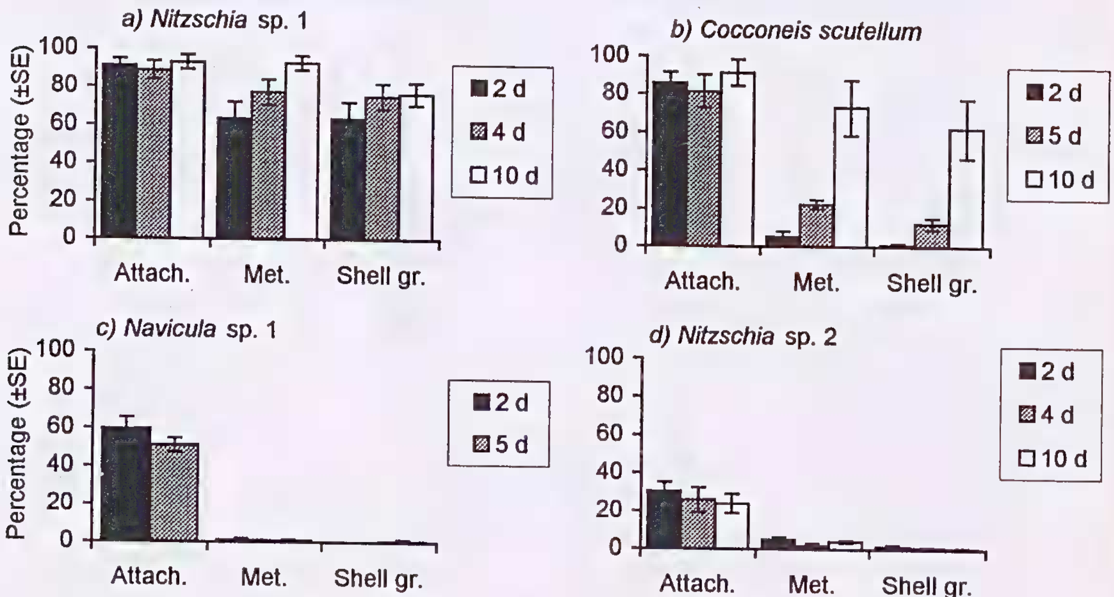

Figure 2. Settlement response of abalone larvae to cultures of selected benthic diatoms, illustrating the range of responses possible. Cultures were not axenic. Graphs a) and d) are for *H.iris* (n=6); b) and c) are for *H. virginea* (n=4). Mean (± SE) diatom densities were as follows: a) $4.0 \pm 0.3 \times 10^5.cm^{-2}$; b) $8.5 \pm 0.6 \times 10^3.cm^{-2}$; c) $1.6 \pm 0.1 \times 10^5.cm^{-2}$; d) $1.4 \pm 0.1 \times 10^5.cm^{-2}$. Attach. = attachment, Met. = metamorphosis, Shell gr. = shell growth. Legend gives number of days of incubation at time of counting.

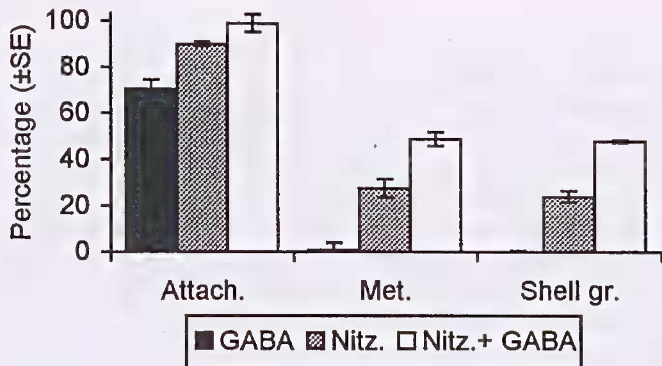

Figure 3. Settlement response of *H. virginea* to combined settlement cues. Combination of GABA (1 μM) plus *Nitzschia* sp. 1 ($1.1 \pm 0.1 \times 10^5$ diatom cells.cm^{-2}, not axenic) produced a synergistic effect which increased percent metamorphosis and shell growth of *H. virginea* after 5 days. Attach. = attachment, Met. = metamorphosis, Shell gr. = shell growth. n = 4.

The larval settlement response to CCA was consistent over multiple experiments with *H. virginea*, although percentage shell growth showed moderate variability. The response to CCA was also consistent for *H. iris* – two previous experiments produced 98–100% attachment and metamorphosis after 2 days of incubation (data not shown).

The larval response to GABA was qualitatively consistent, but showed some quantitative variation. In particular, metamorphosis of *H. iris* and attachment of *H. virginea* varied between experiments (Table 3). The variation in settlement response was not related to larval development (as indicated by rows of radula teeth in Table 3). Percent attachment of *H. iris* was always high, and did not parallel variations in metamorphosis. The distinction between the response of the two abalone species to GABA (i.e., *H. virginea* attached but failed to metamorphose) was maintained across multiple experiments.

FSW was a valid negative control, producing consistently low attachment and virtually no metamorphosis or shell growth.

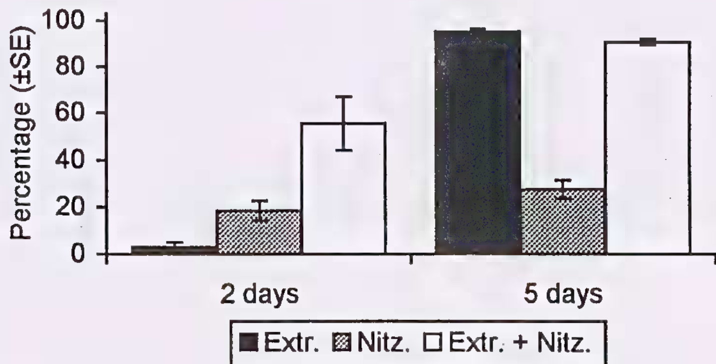

Figure 4. Metamorphosis of *H. virginea* in response to combined settlement cues. Combination of a crustose coralline algae extract ("Extr.", 0.44 mg/ml) and *Nitzschia* sp. 1 ("Nitz.", $1.1 \pm 0.1 \times 10^5$ diatom cells.cm^{-2}, not axenic) produced a synergistic effect which accelerated metamorphosis of *H. virginea*. X-axis labels show number of days of incubation at time of counting. n = 4.

Discussion

Complex response to range of settlement cues

The results demonstrate the complexity of the settlement responses of *H. virginea* and *H. iris*. Complete and rapid metamorphosis was induced only by CCA-coated pebbles and one of the diatom strains tested. The response to all other cues tested varied in either the timing or end-point of the response, or in the proportion of the larvae responding.

Table 3. Comparison of settlement response to selected cues from repeated experiments using standard procedures. Data are mean ± SE, counted after 2 days and 4 days of incubation. "Rows teeth" is the number of chitinized rows of teeth on the radula when the experiment was set up. nd = not determined. n = number of replicate treatments (50–100 larvae per replicate). CCA = crustose coralline algae-covered pebbles; FSW = 1 μm filtered, UV treated natural seawater. Attach. = attachment, Met. = metamorphosis, Shell gr. = shell growth.

		2 day counts			4 day counts			
Spawning date	Rows teeth	% Attach.	% Met.	% Shell gr.	% Attach.	% Met.	% Shell gr.	n
CCA – *H. virginea*								
15/7/96	9	95 ± 2.4	89 ± 3.7	74 ± 4.0	nd	nd	nd	5
29/5/96	10	95 ± 0.7	91 ± 1.6	81 ± 9.6	nd	nd	nd	3
17/4/96	10	98 ± 1.0	96 ± 0.4	87 ± 5.1	nd	nd	nd	2
6/5/95	7	98 ± 1.0	95 ± 0.9	58 ± 15.5	nd	nd	nd	3
27/4/95	8	96 ± 1.0	92 ± 4.4	71 ± 15.4	nd	nd	nd	3
27/10/94	7	83 ± 3.2	78 ± 3.9	49 ± 9.7	nd	nd	nd	3
4/10/94	7	100 ± 0.3	97 ± 1.1	50 ± 9.4	nd	nd	nd	5
Mean±SE		95 ± 2.1	91 ± 2.4	67 ± 5.7				
1 μM GABA – *H. virginea*								
15/7/96	9	65 ± 10.5	3 ± 1.9	0 ± 0	nd	nd	nd	5
29/5/96	10	64 ± 4.2	0 ± 0	0 ± 0	nd	nd	nd	3
17/4/96	10	71 ± 3.2	0.9 ± 0.9	0 ± 0	55 ± 7.2	1.0 ± 0.9	0 ± 0	2
6/5/95	7	88 ± 2.3	0 ± 0	0 ± 0	92 ± 1.0	0.5 ± 0.5	0 ± 0	3
27/4/95	8	81 ± 1.8	0 ± 0	0 ± 0	93 ± 1.7	0.4 ± 0.4	0.4 ± 0.4	3
27/10/94	7	65 ± 6.4	0 ± 0	0 ± 0	65 ± 8.9	1.4 ± 1.4	0 ± 0	3
4/10/94	7	96 ± 1.4	17 ± 2.2	0 ± 0	95 ± 1.7	39 ± 4.5	0.4 ± 0.4	5
Mean±SE		76 ± 4.8	3 ± 2.4	0 ± 0	80 ± 8.3	8 ± 7.6	0.2 ± 0.1	
FSW – *H. virginea*								
15/7/96	9	1.4 ± 0.2	0.3 ± 0.2	0 ± 0	nd	nd	nd	5
29/5/96	10	8.6 ± 3.3	0 ± 0	0 ± 0	nd	nd	nd	3
17/4/96	10	0 ± 0	0 ± 0	0 ± 0	1.1 ± 1.1	0 ± 0	0 ± 0	2
6/5/95	7	12 ± 0.5	0 ± 0	0 ± 0	19 ± 7.1	0 ± 0	0 ± 0	3
27/4/95	8	14 ± 2.6	0 ± 0	0 ± 0	9 ± 4.4	0 ± 0	0 ± 0	3
27/10/94	7	13 ± 2.1	0 ± 0	0 ± 0	8 ± 4.1	0 ± 0	0 ± 0	3
4/10/94	7	17 ± 3.3	0.3 ± 0.3	0 ± 0	9 ± 4.4	0 ± 0	0 ± 0	5
Mean±SE		9 ± 2.5	0.1 ± 0.1	0 ± 0	9 ± 2.8	0 ± 0	0 ± 0	
1 μM GABA – *H. iris*								
14/08/96	9	96 ± 0.8	36 ± 1.9	31 ± 0.5	93 ± 1.2	90 ± 1.9	84 ± 1.4	2
8/11/95	8	98 ± 0.8	20 ± 2.5	8 ± 2.2	87 ± 3.7	39 ± 4.8	33 ± 4.0	5
8/11/95	12	96 ± 0.7	44 ± 8.1	16 ± 2.7	90 ± 2.1	58 ± 5.7	37 ± 8.8	4
15/09/94	9	92 ± 2.3	61 ± 2.9	7 ± 2.2	nd	nd	nd	5
21/10/93	9	98 ± 2.4	60 ± 1.6	40 ± 4.3	84 ± 0.6	71 ± 6.5	56 ± 1.5	2
Mean±SE		96 ± 1.1	44 ± 7.7	20 ± 6.5	89 ± 1.9	65 ± 10.7	53 ±11.6	

The complexity of the settlement response in *H. iris* and *H. virginea* contrasts with the relatively clear cut results obtained during extensive studies with *H. rufescens* Swainson 1822 by Daniel Morse's group. Under very similar experimental conditions to ours, Morse's group found that larval settlement was either complete and rapid, or absent (*e.g.*, Morse and Morse 1984). In contrast, hatchery studies with *H. rufescens* have produced some conflicting findings (Searcy-Bernal *et al.* 1992; Slattery 1992) and studies of other marine invertebrates (Rodriguez *et al.* 1993) include examples of intermediate settlement responses, such as we describe here.

Complete and rapid settlement in response to intact coralline algae is common to *H. rufescens* (Morse and Morse 1984), *H. iris*, *H. virginea* (this study), and many other marine invertebrates (Rodriguez *et al.* 1993). Beyond this commonality, some differences in settlement behaviour among the three abalone species are evident. *H. iris* and *H. virginea* showed very similar settlement responses (except to GABA – Figure 1), but they differed in several ways from *H. rufescens*.

In *H. rufescens* plantigrade attachment was described as "an accurate indicator of metamorphic commitment" (Yool *et al.* 1986: 258). In conditions equivalent to our FSW negative control, 99–100% of *H. rufescens* larvae continued to swim (Morse and Morse 1984). By contrast, for *H. iris* and *H. virginea*, some cues induced attachment without subsequent metamorphosis (Figure 1f–h, Figure 2b) and our FSW controls typically had 10–15% attachment but virtually no metamorphosis. Attachment was reversible in *H. iris* and *H. virginea*, as seen in GABA, KCl and FSW treatments (Figure 1).

Toxicity of CCA extracts to abalone larvae was reported by Morse and Morse (1984). They found that non-toxic doses of the same extract induced metamorphosis and peristomal shell growth within 36 hours. In contrast, we observed delayed and gradual metamorphosis of *H. virginea* and *H. iris* in response to CCA extracts (Figure 1), and observed minimal peristomal shell growth in the presence of extracts (Table 2). This effect was reduced with lower doses of partially purified extract, suggesting that fully purified inducer compound(s) would not interfere with post-larval development.

Neither *H. iris* nor *H. virginea* responded to 1 μM GABA as convincingly as *H. rufescens* (Morse 1984). There was also a clear distinction between the response of *H. iris* and *H. virginea* to 1 μM GABA, with *H. virginea* showing attachment but not metamorphosis. Morphologically incomplete settlement in response to GABA has been noted for some other marine invertebrate larvae (Rodriguez *et al.* 1993). Excess KCl induced a similar "attachment-only" response for *H. iris* and *H. virginea* in the present study. This is surprising given that many marine invertebrates metamorphose when exposed to this cue (Rodriguez *et al.* 1993). Variations of our bioassay chamber type and antibiotic treatments (to more closely match previous studies) did not alter our results for KCl or GABA.

Our results were in general agreement with previous data for *H. iris*. Moss and Tong (1992) showed strong settlement of *H. iris* larvae on CCA-coated rocks. However, their inferred settlement rate for *H. iris* in response to 1 μM GABA, "diatom film", and "GABA + diatom film" (means of 26%, 45% and 49% respectively after 48 h) were considerably lower than in the present study.

Our results showed a wide range of responses to different diatom cultures. Kawamura and Kikuchi (1992) reported a similar range of responses to diatom cultures with *H. discus hannai* Ino 1953. In contrast, Morse's group found no settlement of *H. rufescens* larvae in response to diatoms or other microbial films (Morse *et al.* 1980) although diatom films are used successfully in hatcheries to induce settlement of *H. rufescens* and many other abalone species (Searcy-Bernal *et al.* 1992).

Source of chemical inducers

Our results show that *H. iris* and *H. virginea* can undergo rapid and complete metamorphosis in response to certain diatom cultures (Figure 2a), so there is no absolute requirement for a CCA-associated inducer. The inducing activity of the diatom culture could derive from diatoms and/or associated microbes such as bacteria. Johnson and Sutton (1994) demonstrated that bacteria mediate the induction of starfish larval settlement on CCA. Since both the CCA and the CCA extracts tested in the present study included their biofilm, our results do not clarify whether the inducer(s) originate from the CCA or associated microflora.

Variability among experiments

The response to a given cue was qualitatively consistent across multiple experiments, but there was some variation in the proportion of the larvae responding. This variation was markedly greater for GABA, than for FSW or CCA (Table 3). The variability would not give rise to false positives or negatives regarding settlement inducers, but could influence the detection of differences in settlement rates between treatments. Such inter-experiment variability may arise from subtle differences between batches of larvae (either from parental influence or environmental conditions during rearing) or through unintentional variations in treatments.

Implications for abalone culture

The complex and varied settlement response documented in this study has implications for abalone culture. Larvae can not be assumed to have metamorphosed when they are no longer swimming. Some settlement surfaces may trigger attachment only, or may require several days to induce high percentage metamorphosis. Fast-acting cues would allow water flow and aeration to be restored promptly to maintain high water quality. There appears to be potential to increase and accelerate settlement by using combinations of cues (Figures 3, 4).

The "attachment-only" cues (Figure 1f–h, Figure 2c) may have application in abalone reseeding programmes based on larval release (e.g., Tong *et al.* 1987). Larvae will be released in areas of suitable habitat to optimise settlement and post-larval survival. Protracted swimming could lead to larvae being advected away from the chosen site to less suitable habitats. If the larvae were primed with an attachment inducer then they would be more likely to settle rapidly to the seafloor, where CCA would trigger metamorphosis.

Separate cues for attachment and metamorphosis?

The fact that attachment can be induced without metamorphosis shows that these events can be uncoupled, and suggests that metamorphosis may require a cue which is separate from, or additional to, the cue for attachment. This possibility has been raised for other marine invertebrates (Hadfield and Pennington 1990; Mokady *et al.* 1992) but has received little attention. Identification of settlement-inducing chemicals from the CCA-biofilm complex (Morse 1992) would help clarify the nature, and number, of settlement cues.

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